

# Overview of Cell Culture Technology

<i>Cell Culture Engineering</i> .....	1
<i>Cell Culture Products and Manufacturing Products</i> .....	3
Biosimilars or Follow-on-Biologics .....	7
<i>Alternative Technologies</i> .....	9
<i>Product Quality and Process Robustness</i> .....	12
Critical Feature of rDNA proteins from mammalian cells .....	12
Protein Product Quality Issues .....	13

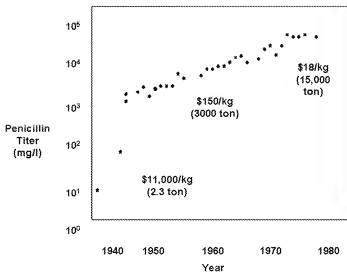
## Cell Culture Engineering - Where we have come from and where we are headed

In the past decade, we have seen continuing growth in mammalian cell culture bioprocessing, primarily driven by the expanding antibody production industry. This expansion is not only in the number of products now available to needy patients, but also in the quantity of antibody produced. Further raising the excitement level is the growing number of prospective therapeutic products for diseases that are likely to become treatable. Although it has been over two decades since the employment of mammalian cell culture in recombinant therapeutic production, and a decade since cell culture bioprocessing was proclaimed to be a "mature technology," the new surge in the quantities of products required and the phenomenally high investment cost for a manufacturing plant have spurred a new drive to enhance cell culture bioprocess technology. Recombinant therapeutic proteins have made a major headway in healthcare. Their future societal impacts may rival those of antibiotics, whose discovery and clinical applications have heralded the arrival of modern medicine. Like microbial fermentation technology in the 1950s, which allowed penicillin to become widely available, advances in cell culture processing technology have been instrumental in making these new medicines available to patients.

When assessing the biotechnological potential of process enhancement, it is instructive to look back at the history that preceded major biotechnological drugs.

### EXHIBIT O

From its discovery by Sir Alexander Flemming and its expansion into clinical applications by Edward Penley Abraham until the end of its second decade, both the product titer and the production volume of penicillin increased almost exponentially. This was followed by a steady but slower enhancement in process, in terms of the product titer, in the following half a century. The roughly three orders of magnitude increase in production volume and product concentration was the result of decades of relentless effort by process scientists and engineers through strain improvement, media development and many other process innovations. Oxygen and heat transfer capacity and mixing characteristics have steadily pushed upward the performance level. On-line sensors, sterility control, equipment reliability and process control, all enhanced drastically over the past decades. However, the success of process technology eventually drove down the price. Penicillin G is no longer produced in the United States, as the cost of production is dramatically lower in some other parts of the world.



With some imagination, one may see a close resemblance of cell culture products to the historical graph of penicillin. Nearly two decades after their first introduction as therapeutic biologics, we have seen process technology make large manufacturing processes a reality, with a steady increase in titer, from tens of milligrams per liter in hybridoma cultures in the 1980s, to four or five grams per liter for some immunoglobulin products today. Although little published information is available, the production cost has also reduced by at least an order of magnitude since the beginning of cell culture products. If one were to plot such a graph for cell culture products for one or two decades from now, we are likely approaching the end of the rapid exponential stage in terms of product titer and process economics. However, in terms of real quantity, tremendous process enhancement was accomplished even after the initial rapid growth phase was over. The question for bioprocess scientists and engineers to ponder is what will it take for cell culture processing to accomplish what the antibiotics industry has achieved in our society in making major medicines affordable to the world's population.

What bioprocess scientists and engineers possess today that was not available to antibiotic researchers or even to the initial innovators in cell culture processes is the ready availability of genomic exploration and cell engineering tools. These new genome-wide investigative tools will greatly facilitate the discovery of genes crucial to conferring cells with desired growth and production characteristics. The new methodology of targeting

host genes for modulation of their expression level leading to altered cell physiology will result in greater productivity as well as increased robustness of the process. Will also facilitate the expansion of biosimilars (Follow-on) and make many medicines available to needy patients around the world at an affordable cost.

## Cell Culture Products and Manufacturing Products

### Principal Viral Vaccines Used in Prevention of Human Virus Diseases

Disease	Source of vaccine	Condition of virus
Poliomyelitis	Tissue culture (human diploid cell line, monkey kidney)	Live attenuated
Measles	Tissue culture (chicken embryo)	Live attenuated
Mumps	Tissue culture (chicken embryo)	Live attenuated
Rubella	Tissue culture (duck embryo, rabbit, or human diploid)	Live attenuated
Smallpox (vaccinia)	Lymph from calf or sheep (glycerolated, lyophilized)	Live vaccinia
Smallpox (vaccinia)	Chorioallantois, tissue cultures (lyophilized)	Vaccinia
Yellow fever	Tissue cultures and eggs (17D strain)	Live attenuated
Influenza	Highly purified subunit forms of chicken embryo allantoic fluid (formalinized UV irradiated)	Inactivated
Influenza	Cell culture (MDCK, Vero)	Attenuated
Rabies	Duck embryo or human diploid cells	Inactivated
Adenovirus	Human diploid cell cultures	Live attenuated
Japanese B encephalitis	Mouse brain (formalinized), cell culture	Inactivated
Venezuelan equine encephalomyelitis	Guinea pig heart cell culture	Live attenuated
Eastern equine encephalomyelitis	Chicken embryo cell culture	Inactivated
Western equine encephalomyelitis	Chicken embryo cell culture	Inactivated
Russian spring - summer encephalitis	Mouse brain (formalinized)	Inactivated
Hepatitis A	Cell Culture MRC-5	Inactivated
Rotavirus	Multivalent bovine	Cell Culture
Varicella Zoster (Chicken Pox)	Cell Culture MRC-5	Inactivated

### Therapeutic Protein Biologics Produced in Non-Mammalian Host

	Activity/Use
Granulocyte colony-stimulating factor (Neupogen)	White blood cell growth for Neutropenia
Insulin (Humulin)	Diabetes
$\alpha$ -Interferon (Intron-A)	Anticancer, viral infections
Somatotropin [human growth hormone] (Humatrope)	Growth deficiencies
Somatotropin [human growth hormone] (Protopin/Nutropin)	Growth deficiencies
Interleukin-2 (Proleukin)	Kidney Cancer

### Non-Antibody Product

Trade name	Type	Therapeutic Use	Manufacturer	U.S. approval year	Host
Aidurazyme	Laronidase	Mucopolysaccharid-eosis I	Genzyme	2006	CHO
Cerezyme	$\beta$ -glucocerebrosidase	Gaucher's disease	Genzyme	1994	CHO
Myozyme	$\alpha$ -galactosidase	Pompe disease	Genzyme	2006	CHO
Fabrazyme	$\alpha$ -galactosidase	Fabry disease	Genzyme	2003	CHO
Naglazyme	N-acetylgalactosamine 4-sulfatase	Mucopolysaccharideosis VI	BioMarin	2005	CHO
Orencia	Ig-CTLA4 fusion	Rheumatoid arthritis	Bristol-Myers Squibb	2005	CHO
Luveris	Luteinizing hormone	Infertility	Serono	2004	CHO
Activase	Tissue plasminogen activator	Acute myocardial infraction	Genentech	1987	CHO

**Therapeutic Antibody Products**

Trade name	mAb type	Therapeutic Use	Manufacturer	U.S. approval year	Host
Orthoclone OKT3	Muromomab CD3	Reversal of acute kidney transplant rejection	Johnson & Johnson	1986	Hybridoma
ReoPro	Abciximab	Prevention of blood clots	Centocor	1994	SP2/O
Rituxan	Anti-CD20 mAb	Non-Hodgkin's lymphoma	Genentech, Biogen IDEC	1997	CHO
Zenapax	Daclizumab	Prevention of acute kidney transplant rejection	Protein Design Labs	1997	NSO
Simulect	Basiliximab	Prophylaxis of acute organ rejection in allogeneic renal transplantation	Novartis	1998	
Synagis	Palivizumab	Prophylaxis of lower-respiratory-tract disease	MedImmune	1998	CHO
Remicade	Anti-TNF- $\alpha$ -mAb	Active Crohn's disease	Centocor	1998	SP2/O
Herceptin	Anti-HER2 mAb	Metastatic breast cancer	Genentech	1998	CHO
Mylotarg	Anti-CD33	Acute myeloid leukemia	Wyeth	2000	CHO
Campath	Anti-CD52 mAb	Chronic lymphocytic leukemia	Millennium, Berlex, Genzyme	2001	CHO
Zevalin	Anti-CD20 murine mAb	non-Hodgkins lymphoma	Biogen IDEC	2002	CHO
Humira	Anti-TNF- $\alpha$ mAb	Rheumatoid arthritis	Abbott	2002	CHO
Xolair	Anti-IgE mAb	Moderate/severe asthma	Genentech	2003	CHO
BEXXAR	Anti- CD20 mAb	Follicular non-Hodgkins lymphoma	GSK	2003	CHO
Raptiva	Anti-CD11a mAb	Chronic psoriasis	Genentech	2003	CHO
Erbix	Chimeric antibody raised against human EGF receptor	EGF receptor-expressing metastatic colorectal cancer	Imclone Systems, Bristol-Myers Squibb, Merck	2004	CHO
Avastin	Anti-VEGF	Metastatic colorectal cancer and lung cancer	Genetech	2004	CHO
Soliris	Antibody binding to C5	Paroxysmal nocturnal hemoglobinuria	Alexion	2007	NSO
Vectibix	Anti-EGFR mAb	Metastatic colorectal cancer	Amgen	2006	CHO

## Industrial Cell Lines

### Major Cell Strains and Lines for Human Biologics Production

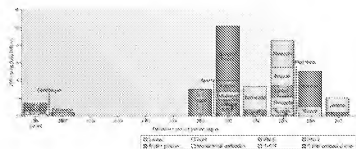
Human Vaccines	
Primary Cells	Green monkey kidney cells (no longer used) Chicken embryo cells
Cell strains	MRC5 (human lung fibroblast)
Cell line	Vero (monkey kidney epithelial cell)
Recombinant Proteins	
<i>Species cell line derived from</i>	
Human	HEK 293, Per C6
Mouse	C-127, NSO, hybridoma cells, SP2/0
Chinese Hamster	CHO
Syrian hamster	BHK

### Cell Lines Used in the Production of Veterinarian Vaccines\*

Vaccines	Cell line
Bovine viral diarrhea virus	MDBK
Bovine parainfluenza virus type 3	MDBK
Bovine rhinotracheitis virus	MDBK
Bovine respiratory syncytial virus	MDBK
Feline leukemia virus	FL72
Feline panleukopenia virus	CRFK
<i>Feline chlamydia</i>	CRFK
Canine parvovirus	CRFK
Canine distemper	Vero
Canine adenovirus type 2	Vero
<i>Ehrlichia canis</i>	DH82
Rabies	BHK-21
Eastern equine encephalitis virus	Vero
Western equine encephalitis virus	Vero
Equine rotavirus	MA104
Equine rhinopneumonitis virus type 1 and 4	Equine Dermal
Equine influenza virus	MDCK
Foot and mouth disease virus	BHK-21
Swine parvovirus	ST, PK
Swine influenza virus	MDCK

\*This table was provided by Terry Ng, 2001. Organisms in italics are intracellular parasitic bacteria.

## Biosimilars or Follow-on-Biologics



Current sales and estimated patent expiry of selected protein products

- ♦ The terms “Biosimilar” or “Follow-on Biologic” refer to products that are marketed after expiration of patents, which are claimed to have similar properties to existing biologic products. Due to the complexity of biologics, a product can only be made that is similar, but not identical.
- ♦ Driving investment in follow-on-biologics is the fact that a number of commercially successful therapeutic proteins will go off patent between 2013-2017, including blockbuster drugs Remicade and Humira
- ♦ A difficult question is how much similarity does a biosimilar have to show to its reference product.
- ♦ While a biosimilars approval pathway has been established in Europe, US has yet to lay down any guidelines.
- ♦ Sandoz was the first company to launch a biosimilar-human growth hormone Omnitrope in both Europe and the United States.

### Approved Biosimilars in the EU

Generic Name	mAb type	Manufacturer	Reference product	Launch
Abseamed	Recombinant human EPO-α	Medice Arzneimittel Putzer (Germany)	Eporex/Erypo (epoetin α Janssen-Cilag, Beerse, Belgium)	2007
Binocrit	Recombinant human EPO-α	Sandoz (Austria)	Eporex/Erypo (epoetin α Janssen-Cilag, Beerse, Belgium)	2007
Epoetin alfa Hexal	Recombinant human EPO-α	Hexal Biotech (Germany)	Eporex/Erypo (epoetin α Janssen-Cilag, Beerse, Belgium)	2007
Retacrit	Erythropoietin zeta	Hospira Enterprises (Netherlands)	Eporex/Erypo (epoetin α Janssen-Cilag, Beerse, Belgium)	2007
Silapo	Erythropoietin zeta	STADA Arzneimittel (Germany)	Eporex/Erypo (epoetin α Janssen-Cilag, Beerse, Belgium)	2007
Omnitrope	Somatropin growth hormone	Sandoz (Austria)	Genotropin (Pfizer)	2006
Vaitropin	Somatropin growth hormone	Biopartners (Germany)	Humantropin (Eli Lilly, Netherlands)	2006

### Marketed Biosimilars in India

Company	Brand Name	Biosimilar	Launch
Ranbaxy	Ceriton	Epoetin	2003
Dr Reddy's	Grastim	G-CSF	2001
	Reditux	MabThera	2007
Wockhardt	Wosulin	Insulin	2003
	Wepox	Epoetin	2001
	Biovac-B	Hepatitis B	2000
Biocon	Insurgen	Insulin	2004
	BioMab-EGFR	MabThera	2006
	Recosulin	Epoetin	2004
Intas Pharmaceuticals	Epoetin, Erykine	Epoetin	2005
	Neukine	G-CSF	2004
Shantha Biotechnics	Shanpoetin	Epoetin	2005
	Shanferon	IFN $\alpha$ 2b	2002
	Shankinase	Strptokinase	2004
	Shanvac B	Hepatitis B	1997

### Marketed Biosimilars in China

Company	Biosimilars
Dragon Pharmaceuticals	Epoetin, filgrastim
Dongbao	Insulin, G-CSF
Anhui Anke Biotechnology	HGH, interferon alpha
Amyotop	G-CSF, IL-11
GeneLeuk Biotech	G-CSF, PEG filgrastim, interferon
Hangzhou Jiuyan Gene	G-CSF, IL-11

### Example of Manufacturing Plants

- Genentech's New Vacaville Facility, California
  - Started construction in 2004, FDA licensure expected 2009
  - Investment: \$800 million
  - Eight 25,000-liter bioreactors
  - Production of Herceptin, Avastin and Rituxan
- Bristol Myer Squibb, Devens, Massachusetts
  - Started construction in 2007, validation in 2011
  - Investment: \$750 million
  - Six 20,000-liter bioreactors, one purification strain
  - Production of Orencia and other biologics
- Biogen IDEC LSM Facility
  - 245,000 ft<sup>2</sup> production
  - Multi-product facility
  - Six 15,000L production reactor capacity



### Dose of some antibody product

Product	Disease Indication	Company	Formulation Configuration
Arneive	Psoriasis	Biogen	7.5mg/0.5ml; 15mg/0.5ml
Enbrel	RA	Amgen	25mg
Heceptin	Breast Cancer	Genentech	440mg/30cc
Humira	Rheumatoid arthritis	Abbott, CAT	40mn (1ml prefilled syringe)
Remicade	Crohn's disease, RA	Johnson & Johnson, Centocor	100 mg/20cc
Rituxan	NHL	Genentech/Iddec	100mg/10cc; 500mg/50cc
Synagis	Respiratory syncytial virus	MedImmune	100mg
Xolair	Allergic Asthma	Genentech/Tanox/Novartis	150mn/5cc

## Alternative Technologies

Mammalian cells are still the main workhorses for biologic production. The other host cells used for biopharmaceutical production include *E.coli* and *Saccharomyces cerevisiae*. Alternative production systems include the following:

- ♦ Insect cell culture
- ♦ Yeast ( *Pichia* )
- ♦ Transgenic animals
- ♦ Transgenic plants

### Insect Cell Culture

Application	Comments
Basic research	Hundreds of genes have been expressed using baculovirus.
Bioproduction	Possibly 25 or more compounds in clinical trials are being produced using baculovirus expression systems.
Gene therapy	A growing number of cell types have been shown to work with BV as the gene-delivery vehicle.
Display systems	Demonstrations of the ability to produce large numbers of proteins suggests BV display systems could lead to combinatorial proteomics.
Bioreagent production	A number of bioreagent suppliers use BV to make target proteins, viral components and other compounds for the research market.
Artificial chromosomes	The large BV genomes suggest use as artificial chromosomes. So far, one patent has been awarded for this application.
Biopesticides	There has been an enduring interest in using BV for biopesticides; many attempts have failed. One of the latest is to use a gene for a scorpion toxin against targeted insects.

### Yeast

Several advances have been made in 'humanizing' the glycosylation characteristics in *Pichia* systems. Glycofi(Merck) has worked towards a multistep genetic engineering process where they first eliminated non-human glycosylation enzymes and subsequently introduced human glycosylation reactions. They currently report a titer of ~ 1.4g/L. If further developed and scaled up, these engineered yeast strains will facilitate batch to batch consistency by producing uniformly glycosylated products.

Product	Company	Use	Status
Medway (recombinant human serum albumin)	Mitsubishi Tanabe Pharma Corporation, Osaka, Japan	Blood expander	On the market in Japan
Hepatitis B vaccine	Shantha Biotechnics Ltd., India	Hepatitis B	On the market in India
Interferon-alpha	Shantha Biotechnics Ltd., India	Hepatitis C/Cancer	On the market in India
DX-88	Dyax Corporation, Cambridge, Mass.	Hereditary angioedema (HAE), a debilitating condition characterized by acute attacks of inflammation.	BLA submitted
Recombinant Human Insulin	Biocon, India	Diabetes, all types	On the market in India
Recombinant collagen	Fibrogen Inc., South San Francisco	Medical research reagents and dermal filler	On the market
Botulism vaccine	USAMRIID/DynPort	Botulism vaccine product	Phase I (U.S.)
Anti-thrombolytic	ThromboGenics Ltd.	Thrombosis Tx	Phase II

## Transgenic Animals

Transgenic organisms for the production of biotherapeutics have been in development for more than a decade. These production systems require low initial capital investment, and have a relatively easy purification process for the glycosylated products. However, so far, only one product has been approved by the FDA- ATryn, produced in transgenic goat milk by GTC Biotherapeutics.

### Animal Species Commonly Used for Expression of Recombinant Proteins in Milk

Species	Reproductive Age (months)	Average # of Offspring	Average Yield per Natural Lactation (l)
Mouse	1	10	0.0015
Rabbit	6	8	1.5
Pig	8	9	120
Sheep	8	2	400
Cattle	15	1	10,000

### Transgenic Animal Products Approved or Under Development

Species	Company	Product	Status	Comments
Goat	GTC Biotherapeutics, MA	ATryn- recombinant human antithrombin-alpha	Approved	Glycosylation patterns differ slightly ( involves N-glycolylneuramic acid- not seen in humans), but was not a regulatory hurdle; Predicted sales of \$6-\$10 million in 2009.
Goat	PharmAthene, MD	Protexia- recombinant human butyrylcholinesterase (BChE)	Development	
Rabbit	Pharming, Netherlands	Rhucin-Recombinant human C.1 esterase inhibitor	Phase 3 trials	For the treatment of hereditary angiodema.
Chickens (eggs)	Origen Therapeutics, Medarex Inc., CA	mAb	Pilot Studies	Functional Mabs produced at 3 mg/egg; some differences in glycosylation; Half life in mouse serum half that of natural antibodies (reduced from 200-100h)

## Product Quality and Process Robustness

### *Critical Feature of rDNA proteins from mammalian cells*

- ◆ Folding and disulfide bond
- ◆ Glycosylation
  - N or O - glycosylation
  - Sulfation or phosphorylation of glycans
  - Affect solubility, clearance and biological activities
- ◆ Other post-translational modifications
  - γ-carboxylation
  - lipidation

#### **Issue Plasminogen Activator (tPA)1**

- ◆ Single polypeptide chain (70 kDa) or proteolytically cleaved at ARG276.
- ◆ Multiple N-linked carbohydrates: ASN117 (high mannose), ASN184 (50% complex multiantennary, 50% unoccupied), THR61 (O linked fucose).
- ◆ Contains 35 cysteine residues, 17 pairs of disulfide bonds. CYS83 can form a disulfide with other free thiols depending upon the growth medium and buffer composition.
- ◆ May form high molecular weight aggregate (complexes with protease inhibitors) and proteolytically cleaved tPA.

#### **Erythropoietin**

- ◆ Contains 40% carbohydrate, only 2 disulfide bonds.
- ◆ 3 N-linked ASN (24,38,83), 1 O linked (SER126) glycosylation sites.
- ◆ O-linked site not essential for in vitro or in vivo activity.
- ◆ Sialic acid residues (average 10 moles/mole Epo) responsible for preserving pharmacokinetic behavior. Muteins lacking 2 or 3 N-linked sites are poorly secreted.
- ◆ N linked glycosylation and sialylation is critical to optimal secretion, structure, in vivo potency.

## Protein Product Quality Issues

### In Process Structural Alternations to Mammalian Protein Biologics

Glycoform	
Site occupancy	Possibly caused by stochasticity of glycosylation process
Altered sialic acid content	
Uncapped galactosyl residue, High mannose	
Glycan distribution out of range	
Amino acid alterations in protein	
Mis-incorporation (coding misreading)	Error rate of amino acid incorporation during translation (1/1000)
Deamidation (Asparagine)	Most likely occurred in culture fluid, may be affected by process conditions, or even product titer
Loss of terminal amino acid <ul style="list-style-type: none"><li>lysine in C-terminus of heavy chain IgG, enzymatic cleavage</li><li>cyclization of N terminus glutamine</li></ul>	
Glycation (addition of reducing sugar (glucose) to amino acids)	
Protein aggregation	
	May be caused by folding in ER or agglomeration in culture or in down stream processing